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Original Article

G Protein Coupled Receptors Potentially Involved in Oligodendrogenesis: A Gene Expression Analysis

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Abstract

Background: Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system characterized by infiltration of inflammatory leukocytes to the CNS followed by oligodendrocyte cell death, myelin sheath destruction, and axonal injury. A logical incidence occurring after demyelination is remyelination. G-protein coupled receptors (GPCRs) activate internal signal transduction cascades through binding to different ligands. This family of receptors are targeted by more than 40% of currently marketed drugs. GPCRs can be successfully targeted for induction of remyelination. GPCRs highly enriched in oligodendrocyte progenitor cells compared to oligodendrocytes are proposed to hamper oligodendrocyte differentiation and therefore their inhibition might induce remyelination. This study aimed to investigate the expression of GPCRs in silico and in vitro.

Methods: We performed gene expression analysis using DAVID and Panther websites on a RNA-seq dataset (GSE52564 accession number). Primary embryonic neural stem/progenitor cell isolation and culture were performed and subsequently NSPCs were characterized by Immunocytochemistry with Anti-Nestin antibody. Expression of *GPR37L1, EDNRB, PDGFRa, CNPase and GFAP* were assessed using real-time PCR. All the experiments were conducted at Shiraz University of Medical Sciences (SUMS), Shiraz, Iran, in the year 2018.

Results: The 14 most highly expressed GPCRs in oligodendrocyte progenitor cells (OPCs) compared to Oligodendrocytes were presented in our study.

Conclusion: The investigation of the most highly expressed GPCRs in OPCs compared to oligodendrocyte in silico and in vitro presents the significant role of GPCRs in remyelination induction. Among the 14 GPCRs mentioned in this study, GPR37L1 is a potential remyelinating drug target and is suggested for further studies.

Keywords: Oligodendrocyte progenitor cells (OPCs); Oligodendrocytes; Remyelination; Demyelination; Gene expression analysis

Introduction

Oligodendrocytes are cells that make and sustain the lipid-rich myelin sheaths insulating and enwrapping axons. During development, these cells arise from oligodendrocyte progenitor cells(OPCs) and neural stem cells (NSCs). In the Central Nervous System (CNS) an insult directed at the oligodendrocytes is the most preliminary cause of demyelination. Demyelination is a condition in which concentric layers of compact myelin, surrounding axons are lost (1). Defects in both oligodendrocyte development and demyelination cause various neurological disorders, such as spinal cord injury and multiple sclerosis (MS) (2, 3). A logical corollary to CNS demyelination is frequently the robust regenerative process of remyelination. The source of remyelination is provided by newly formed oligodendrocytes in adult brain through differentiation of Neural stem/progenitor cells (NSPCs) or OPCs (4).

Switching from OPCs differentiation to oligodendrocytes is mostly halted by various factors in different diseases. To overcome this incident and awaken the sequestered reservoir of remyelination, pharmacological activation of these cells has recently become the center of attention (5). There is the possibility of re-purposing the currently marketed drugs for remyelination. G-protein-coupled receptors (GPCRs) trigger one of the most common response pathways in the cell formed by the guanine nucleotide-binding proteins (G proteins). Approximately half of all drugs currently on the market target these receptors (6). Hunting for new drug targets can be tedious and costly through functional screening of drugs in 2D cell cultures for remyelination. A study published in Nature shed light on the efficacy of two drugs, miconazole and clobetasol in promoting precocious myelination in vivo in early postnatal mouse pups (7). A study discovered Benztropine (a well-established approved drug for the treatment of Parkinson's disease) to be the most effective inducer of OPC differentiation (8). The targets for these proposed drugs are mostly GPCRs.

Rational screening of drug targets using in silico tools is another faster and less costly way for drug target discovery. Studies for functional rational drug/drug target screening for remyelination were lacking. In the current study, we evaluated differential GPCRs expression in oligodendrocytes and OPCs to rationally propose drug targets for remyelination. In an RNA-seq data analysis using panther and David websites in the current study, we investigated the expression level of GPR37L1 a closely related orphan GPCR to GPR37 (9-11). In addition, we derived the most expressed GPCRs in oligodendrocyte progenitor cells compared to mature oligodendrocytes. Several of the found GPCRs have already been linked to remyelination induction. Among the GPCRS, GPR37L1 is a potential drug target for remyelination induction. GPR37L1 is a constitutive expressed orphan GPCR with a distinct expression pattern in glial cells and CNS. The paucity of information on this receptor has cast a shadow over its possible fascinating role in myelination. We proposed GPR37L1 and other mentioned GPCRs as drug targets for remyelination induction and promotion of oligodendrocyte differentiation.

Materials and Methods

Gene Set Enrichment and pathway Analysis

RNA-seq data was downloaded from the website (https://web.stanford.edu/group/barres_lab/brain_rnaseq.html). The accession number from GEO datasets was GSE52564. The data sets were analyzed using DAVID and Panther websites. First, the differential gene analysis following annotation of RNA-seq data were performed. Next, we did a gene set enrichment analysis displaying different gene sets (such as GPCRs and lipid biosynthesis gene sets) upregulated in OPCs and OLs. Finally, we derived the pathway analysis in

both cell types and compared the expression of highly expressed pathways in each cell type.

All these were done according to the published protocols of each website in the journal of Nature protocols (9, 10). All the experiments were conducted at Shiraz University of Medical Sciences (SUMS), Shiraz, Iran, in the year 2018.

Animals, Surgery and Dissection of mice cortices

All mice examined in this study were obtained from Comparative and Experimental Medical Center of Shiraz University of Medical Sciences (SUMS). All animal procedures were conducted following protocols approved by the Animal Ethics and welfare committee of SUMS (no. IR.SUMS.REC.1396.S448). Female and male BALB/c mice were mated at 1:2 ratio (male: female) and female mice were checked for the following next 5 d until vaginal plaque was observed.

On the 14th day of the mouse pregnancy, pregnant female mouse was deeply anesthetized with ketamine and xylazine and sacrificed. 14.5 d old mice embryos' heads were dissected with fine scissors to be used further for isolation of NSCs. The cortex was subtly dissected with fine forceps and scissors under dissecting microscope and placed in ice-cold Phosphate Buffered Saline (PBS) solution containing 10% penicillin-streptomycin (Penstrep).

Primary embryonic neural stem cell Isolation and Culture

Following the dissection procedures described above, we pipetted the cell pellet in 1 ml warm Neurocult proliferation medium and counted the number of viable cells using trypan blue 0.4% dead cell exclusion method(12). 2 $\times 10^5$ viable cells per 1 ml of complete neural stem cell culture medium are cultured in T25 flasks (SPL). For a T25 Flask we cultured the cells with complete medium containing Neurocult proliferation medium and supplement at a (1:9 ratio), 20 ng/ml Epidermal Growth Factor (EGF) and 1% penstrep. Cell cultures were incubated at 37 ºC in 5% CO2 for 5 to 7 d and every 2 d, half of the medium was changed. This was considered passage 0. NSPCs from passages 2-4 were used for the experiments every 5 or 7 d, cells were passaged by tripsinization and then centrifuged at 1200 g for 5 minutes. The single cells were then seeded at 1×10^5 cells/ml in complete medium.

NSPCs characterization by Immunocytochemistry with Anti-Nestin antibody

NSPCs were seeded at a density of 5000 cells/well in poly-l-ornithine coated plates (SPL) in complete neural stem cell proliferation for 7 days. The coating procedure was performed in a way to let neural stem cells form neurospheres; diluted (1:4) Poly-l-ornithine in PBS was incubated in 96 well plates for only less than an hour in an incubator. After 7 days wells were gently washed twice with PBS and fixed in 1% paraformaldehyde for 20 minutes. Fixed cells are then permeablized in 0.1% TritonX-100 for 5 min for nestin (a cytoplasmic marker). Nonspecific antibody binding sites were blocked by incubating with 5% Normal Goat Serum and 1% Bovine Serum Albumin in PBS for 1 h. Cells are rinsed twice with PBS and labeled with mouse nestin monoclonal antibody (1:200) in PBS containing 10% BSA at 4 ºC overnight. Before staining the nuclei with 7-Aminoactinomycin D (7-AAD) nuclear stain for 1 min, wells are rinsed cautiously with PBS and then incubated with FITC-conjugated anti-mouse nestin antibody (1:600) for 2 h at room temperature.

Immunofluorescent-labeled cells were visualized with Nikon Eclipse TS100 microscope coupled with a True Chrome Metrics camera. Images were taken at 10X magnification from 3 random areas in each well (n=3 for each condition). Images were processed using Photoshop CC 2016 and the intensity of each image was analyzed using ImageJ software.

RNA extraction and Real-time PCR

The NSPCs were plated at a density of 2×10^5 Cells per 24 well plates coated with Poly-l-ornithine in complete neural stem cells media. Total RNA was extracted from cultured cells using RNXplus reagent (Cinnagen) according to the manufacturer's instructions.

Total RNA (1μM) was reverse transcribed into cDNA using Prime Script II First Strand cDNA synthesis Kit (TaKaRa), and 0.1% of cDNA mixture was used as polymerase chain reaction (PCR) template. Primers are shown in Table 1. The reaction was performed using a SYBR Green PCR Master Mix Kit (Yektataghiz azama) in a Rotor-gene Q (Qiagen) with an initial denaturation step at 95 ºC for 30 sec, following 45 cycles. Each cycle transitioned between 3 steps of 95 ºC for 5 sec, 60 ºC for 30 sec and an extension step of 72 ºC for 30 sec. Beta Actin was employed as the housekeeping gene to account for sample variability. Relative gene expression is represented as Fold change ($2^{-\Delta\Delta Ct}$).

Table 1: Designed primers for the study

Gene Name	*Forward Primer 5' →3'*	*Reverse Primer 5' →3'*
EDNRB	TCGGACTACAAAGGAAAGCC	TGAACAGCCACCAATCTT
GPR37L1	GTTTGCTGTGGGTATCGTTGG	AGAGACTGAAGGTTGTGACT
PDGFRalpha	GTTGCCTTACGACTCCAGAT	TCACAGCCACCTTCATTACA
CNPase	CTCTACTTTGGCTGGTTCCT	TTCTCCTTGGGTTCATCTCC
GFAP	GAGACAGAGGAGTGGTATCGG	GCTTCGTGCTTGGCTTGG
Actin Beta	GCAACACGCAGCCAC	CGCAGGGATATCGTCATCCA

Software

For fluorescence intensity analysis and Percentage of antibody, positive cells calculation Image J software was used. The total number of cells and antibody-stained cells were counted manually and automatically by Image J software. For the neurosphere assay (estimating the diameter of the Spheres) Infinity Analyze version 4.6 was used. For primer Design Primer-BLAST and Gene runner 6.5.51 was used. Rotor-Gene Q software, ver. 2.3.1 was used to visualize and partially analyze the Real-time PCR results. Raw data were analyzed in Excel 2014.

Statistical Analysis

All experiments were conducted at least in triplicate (n=3). Data were analyzed using Graphpad prims software (ver. 6.0); Data in the figures are expressed as mean ± SEM. Two-way ANOVA followed by Sidak multiple comparison post hoc was performed to compare differences among multiple treatments. Independent samples t-test was performed to compare differences between two conditions. For all experiments significance was defined as P-value<0.05.

Results

The 14 most highly expressed GPCRs in OPCs compared to Oligodendrocytes

In this study, we analyzed the gene expression profiles of 5 different cells. The comparison of gene expression showed a high resemblance in the expression pattern of Gpr17, EDNRB, Gpr56, Gpr19 and GPR37L1 as indicated in Fig. 1. However, EDNRB and GPR37L1 expression patterns are more alike, both being upregulated in OPCs and astrocytes and lower expression in newly myelinating oligodendrocytes. Whereas, their expression in myelinating oligodendrocytes compared to OPCs is merely unnoticeable. Whether GPR37L1 shares a similar function with EDNRB on oligodendrocytes differentiation is quite an interesting dilemma (13, 14). The expression of Chrm1, Chrm2, Hrh1 and P2ry1 was not significant. Gpr37 and Gpr62 have the same expression pattern too but it was not significant to be discussed.

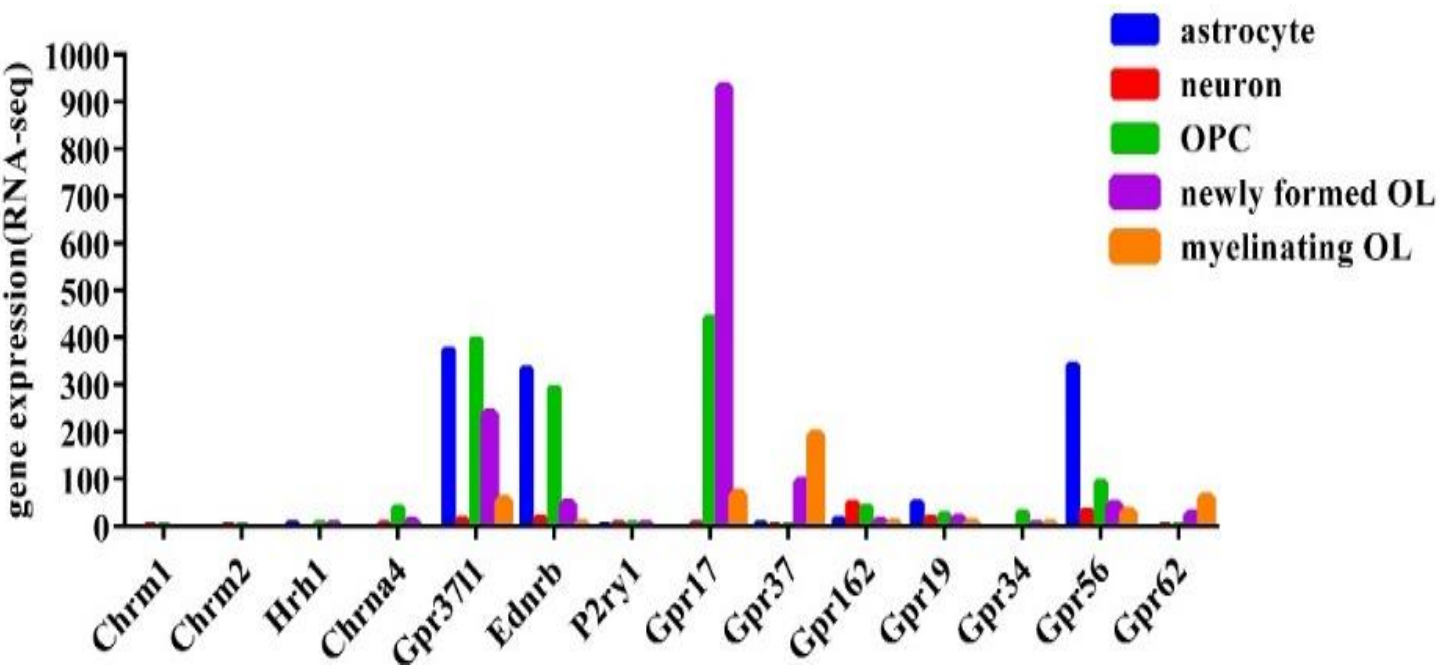

Fig. 1: Comparison of GPCRs gene expression profiles in astrocyte, neuron, OPC, newly formed OL, myelinating OL cells

Primary culture of Nestin-positive NSPCs and further passaging of these cells

Primary NSPCs culture formed neurospheres of > 200 μM in diameter (P0) after 7 d of culture. The expression of Nestin decreased (data not shown) and cell culture time was shortened to ~ 5 d in the following passages (Fig. 2). The percentage of PDGFR-alpha mRNA expression didn't show a significant increase in passage 4 compared to passage 2 of cortical neural stem cells culture, albeit CNPase showed a noticeable fold change expression of 1.66 and GFAP mRNA expression was downregulated by almost 5 fold change (50%). PDGFR-alpha is a marker for oligodendrocyte progenitor cells, CNPase is upregulated in mature pre-myelinating and myelinating oligodendrocyte, expressed along MBP and PLP constituents of myelin sheath. As commonly known GFAP is an astrocyte marker, but is also abundantly expressed in radial glial cell (which is the progenitor cell giving rise to both OPCs and astroglial cells) (Fig. 2).

Endothelin B Receptor and GPR37L1 receptor relative expression in NSPCs at mRNA level

Eendothelin B and GPR37L1 expression exhibited greatly increased expression during further Cortical NSPCs passages (with EDNRB and GPR37L1 gene expressions enhanced by almost 3 and 6 fold change in cortices derived from passage 4 compared to passage 2) as shown in Fig. 2. EDNRB and GPR37L1 expression were explored in NSPCs derived from embryonic cortices compared to ganglionic eminences (both at passage 02) alongside Glial markers (CNPase and PDGFR-alpha) and astrocytic marker GFAP. Both EDNRB and GPR37L1 displayed higher gene expressions in NSPCs derived from cortices compared to ganglionic eminences.

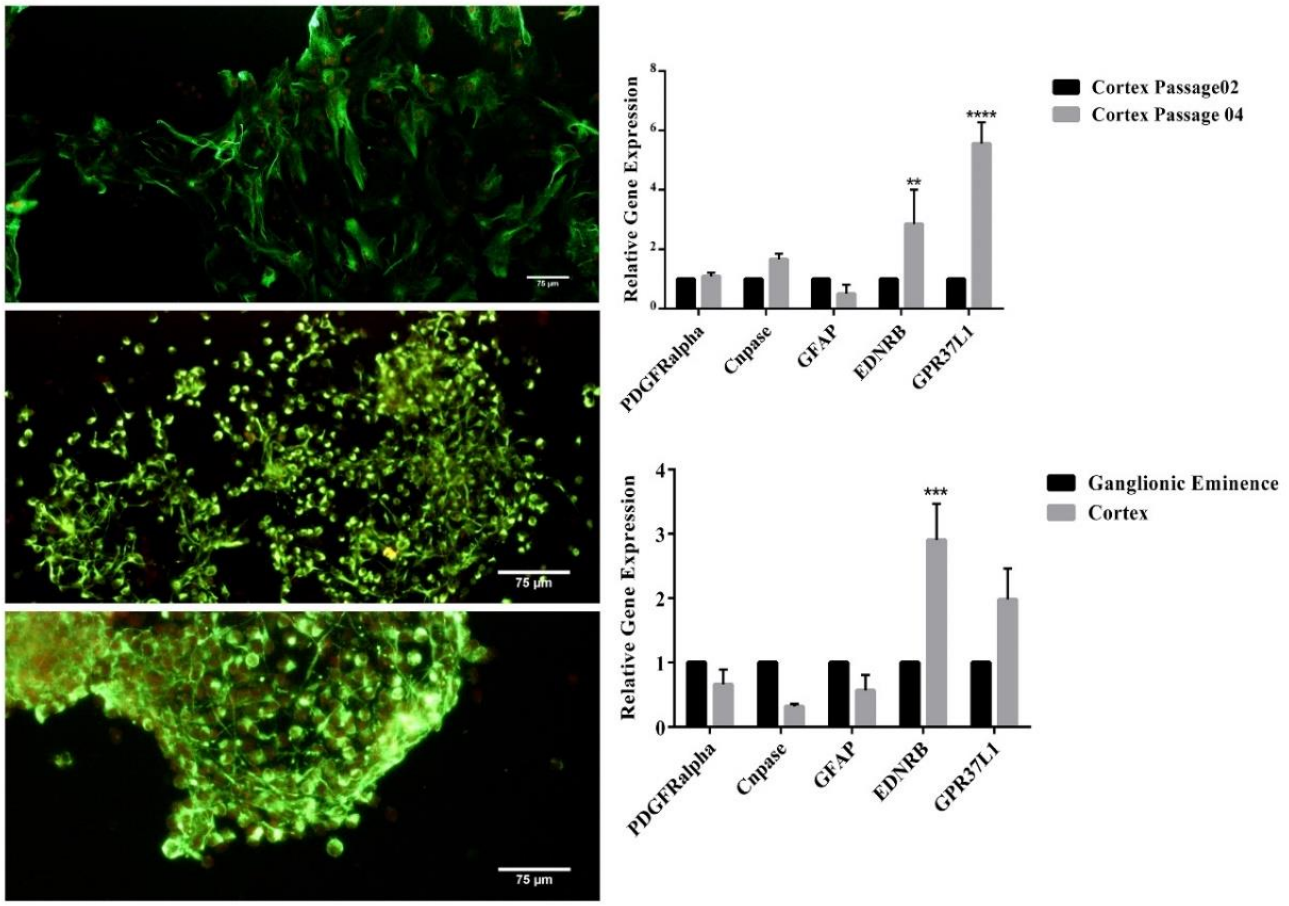

Fig. 2: Left side: Primary culture and further passages of Nestin-positive NSPCs
Right side: Relative expression of Endothelin B Receptor and GPR37L1 receptor in NSPCs at mRNA level

Discussion

In this study, we presented the possible role of GPCRs in driving remyelination. We conducted gene expression analysis on a RNA-seq dataset of genes expressed in cells derived from mouse nervous system. The expression of GPCRs highly expressed in OPCs compared to oligodendrocytes are presented here. Most of these genes were previously reported to harm remyelination.

Among these GPCRS, expression of EDNRB and GPR37L1 were investigated in vitro as well. Anti-Nestin staining was performed for characterizing NSPCs. Commonly Nestin has been utilized as a biological marker to identify NSCs (15). Cells express nestin early in their life cycle. Nestin expression is down-regulated as the cells progress down a specific cell lineage to become either neuron or glial cells (16, 17). NSPCs express nestin and further passages of cortical NSPCs show a slight reduction in Nestin expression (data not shown). The expression of Gpr37l1and Ednrb was explored at mRNA level using real-time PCR in conseqeutive passages of cortical NSPCs compared to Ganglionic eminences derived NSPCs. Our finding shows Gpr37l1 has a similar pattern of expression in mice compared to Ednrb.

Developmentally, oligodendrocytes arise from OPCs (18, 19). OPCs themselves arise from subventricular cells in the brain and spinal cord. A myriad of different permissive and inhibitory factors orchestrate the differentiation of oligodendrocytes. Several Inhibitory factors are expressed by axons to usher myelination and diffe-

rentiation of OPCs. Induction of remyelination can be addressed by activation of endogenous OPCs present around demyelinated lesions (2, 3, 20, 21). Our premise was that GPCRs highly expressed in OPCs compared to oligodendrocytes cast an inhibitory effect on oligodendrocyte differentiation.

The most highly expressed GPCR genes in OPCs compared to mature oligodendrocytes were Gpr37l1, Ednrb, Gpr17, Gpr37 and Gpr56. Ednrb, Gpr17 and Gpr37 roles have all been investigated in the context of remyelination. Ednrb Regulates the Rate of Oligodendrocyte Regeneration during Remyelination. Other GPCRs such as Chrm1, Chrm2 and Chrna4 receptors belong to the family of muscarinic and cholinergic receptors and they all demonstrate a significant and mostly impermissive role in oligodendrocyte differentiation and remyelination.

GPR17 is a P2Y purinergic GPCR affecting oligodendrocyte differentiation and myelination. GPR17 cast a negative effect on this phenomenon. The absence of Gpr17 enhances remyelination and the activation of Erk1/2 pathway following Gpr17 down-regulations corroborate this finding (22, 23). An article released in 2009 portrayed the role of Gpr17 in remyelination, downregulated in Olig1-null mice(24). Recently identification of a non-specific antagonist called pranlukast accelerated myelination following toxin-mediated demyelination (25). Endothelin B receptor was recognized as a potential inhibitory drug target that works in a paracrine and autocrine way to inhibit OPCs differentiation. ETBR is expressed on both astrocytes and OPCs(26). Coupling of ET-1 ligand to this receptor on astrocytes promotes Notch activation in OPCs during remyelination through induction of Jagged 1 expression in reactive astrocytes (13, 14).

In a microarray-based experiment of isolated cells, GPR37 was shown to be strongly enriched in mature and pre-mature oligodendrocytes (27). GPR37 mutant mouse exhibits premature oligodendrocyte differentiation, precocious myelination and hyper myelination (28). The mechanism by which GPR37 regulates multiple stages of myelination is elusive. Nevertheless, given its strong enrichment in oligodendrocyte lineage, it can be an interesting drug target. GPR37 is structurally closely related to endothelin B receptor. GPR37 is an orphan GPCR distinctly expressed in neuronal and glial cells of the CNS. A negative regulatory effect of GPR37 was manifested on oligodendrocyte differentiation and myelination (29-31). GPR37 and Gpr37l1 act as parkin substrates. They are expressed in different CNS areas. The absence of these receptors caused an increase in ERK1/2 phosphorylation in both cultured oligodendrocytes and leads to decreasing myelin growth (29, 32). Moreover, lacking GPR37 showed changes in the expression of oligodendroglial proteins such as myelin associated glycoprotein (MAG) (33). A highly desirable property of a druggable target is its tissue or cell-type specific expression, reducing the concern over unwanted effects. GPR37L1 is an orphan GPCR exclusively expressed in the nervous system and are known to be expressed on both neurons and glial cells (30). Another closely related GPR37L1 is called GPR37 or also known as parkin associated endothelin-like receptor or "Pael-R". the suggested cognate ligand for these two receptors is prosaposin which is still under investigation. Prosaposisn and prosaptide have been numerously reported to exert neuroprotective and oligoprotective effects. Through a bioinformatics approach, several surrogate ligands were proposed to inhibit GPR37l1, one of them is an orexin 2 receptor antagonist called JNJ10397049 (34).

Conclusion

The investigation of the most highly expressed GPCRs in OPCs compared to oligodendrocyte in silico and in vitro presents the significant role of GPCRs in remyelination induction. Among the 14 GPCRs mentioned in this study, GPR37L1 is a potential remyelinating drug target and is suggested for further studies. Other GPCRs presented in this study (Gpr56, Gpr62, Gpr19, Gpr162 and Hrh1) have a probable role in remyelination

induction as well and are presented for further analysis and experimentation in vitro and in vivo.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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